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13. ABSTRACT (Maximum 200 Words)

The goal of this project is to establish an animal model of plexiform neurofibroma which then can be used to test emerging anticancer therapies for the management of tumor growth in NF1 patients. The first specific aim is to establish a syngraft model of NF1 tumorigenesis. Progress toward this aim includes: a) established and characterized numerous Schwann cell cultures from wild-type and heterozygous and homozygous and homozygous null NF1 knockout mouse embryos; b) documented several lines of evidence that Nf1-/- Schwann cell have a growth advantage in vitro; c)effectively transfected Nf1-/- Schwann cell cultures with AAV-GFP; d)implanted GFP-labeled Nf1-/- cells in mouse nerve and detected them with GFP immunohisto-chemistry; e) documented tumorigenic growth by Nf1-/- Schwann cells implanted in the nerves of adult mice and; f) established a highly relevant syngraft model by implanting $Nf1^{-/-}$ Schwann cell into mice with and Nf1 background. The second aim is to establish a xenograft model to examine the growth of human neurofibroma tumor cells. Progress toward this aim includes: a) established a breeding colony of Nf1-/+ scid mice to be used as hosts for tumor grafts; b)achieved sizeable tumors by engrafting human NF1 Schwann cells in mouse nerves; c)provide a thorough characterization of human NF1 schwann cells cultures and their tumorigenic properties; d) achieve NMR methods to access tumor growth within the mouse nerve and; e)assess tumorigenic growth and neovascularization by engrafted NF1 tumor cells.

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INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common genetic disease characterized by a high incidence of neurofibroma, a nerve tumor composed predominantly of Schwann cells. The NF1 condition is characterized by a germline defect in one NF1 allele (NF1 $^{-/+}$) whereas a homozygous NF1 deficiency (NFI^{-1}) is lethal in utero. It is generally believed that neurofibromas arise due to a constitutional mutation of the second NF1 allele that imparts abnormal growth of (NF1^{-/-}) Schwann cells. The goal of this project is to establish an animal model of plexiform neurofibroma which then can be used to test emerging anticancer therapies for the management of tumor growth in NF1 patients. The first specific aim is to establish an allograft mouse model of NF1 tumorigenesis. To mimic the conditions believed to underlie neurofibroma formation, Schwann cell cultures will be isolated from embryonic Nf1^{-/-} mice (prior to lethality). Nf1^{-/-} Schwann cell cultures will be prelabeled with a tracer protein and then implanted into the nerves of young NfI^{-/+} mice. The grafts will be examined to see if neurofibroma-like tumors are formed. These experiments will test the hypothesis that Nf1^{-/-} Schwann cells are the cause of neurofibromas and will determine if heterozygous nerve cells (with the NfI^{-/+} background) might also contribute to the formation of these tumors. The second aim is to establish a xenograft mouse model to examine the tumorigenic growth of human neurofibroma-derived Schwann cells (some of which have additional genetic defects). Schwann cell cultures established from human plexiform neurofibromas will be transplanted into the nerves of immunodeficient $Nf1^{-/+}$ mice (to preclude immunorejection). Tumor formation will be assessed histologically and key features compared to naturally-occurring neurofibroma. including cell proliferation, nerve invasion and formation of new blood vessels. These experiments will test the hypothesis that human plexiform neurofibroma-derived Schwann cells are tumorigenic and that the transplantation model represents a valid model to study NF1 tumor development and biology. This work will also investigate and optimize methods to monitor neurofibroma growth in the living animal by magnetic resonance imaging (NMR). These efforts will increase the usefulness and clinical relevance of the NF1 mouse nerve model and should provide an effective means to examine plexiform tumor growth and response to therapies in the living animal.

BODY

Technical Objective 1: Establish a Valid Syngraft Mouse Model of NF1 Tumorigenesis.

Task 1: Establish and label embryonic *Nf1* knockout mouse Schwann cell cultures:

Progress:

We have tested numerous approaches to the culture of Schwann cells from day 12.5 embryos. The best differentiation and expansion of Nf1 Schwann cell cultures was accomplished by co-culture with ganglionic neurons and later treatment with glial growth factor-2 (GGF2). These methods yielded highly enriched cultures of Schwann-like cells which express the Schwann cell antigens p75 and S-100 (see Annual Report 2000-01). We next compared the tumorigenic properties of $NfI^{-/-}$, $NfI^{-/+}$, and $NfI^{+/+}$ Schwann cells using in vitro assays for proliferation and apoptosis. Results showed that proliferation by $NfI^{-/-}$ Schwann cells is nearly double that of wild-type cells, and heterozygous cells showed intermediate proliferation. Also, proliferation by $NfI^{-/-}$ Schwann cells was growth factor independent and was not further increased by elevated serum or addition of GGF2 (see Annual Report 2001-02). On the other hand, proliferation by $NfI^{-/+}$ and $NfI^{+/+}$ Schwann cells was increased significantly by elevated serum and glial growth factor-2, but even with mitogen stimulation neither genotype achieved the mitotic rates of

the $NfI^{-/-}$ Schwann cells. TUNEL assays showed that apoptosis was fairly low (6-8% of cells) in all the cultures grown in standard culture conditions and was not altered significantly by high serum or GGF2. Based on these in vitro data, we predicted that $NfI^{-/-}$ Schwann cells may have a greater tumorigenic potential.

Our next goal was to examine the tumorigenic growth of NfI Schwann cells in vivo. It was necessary to prelabel the mouse NfI Schwann cell cultures to later identify them when implanted in the host mouse nerves. We succeeded in labeling the mouse Schwann cells cultures in vitro with a green fluorescent protein (GFP) adeno-associated virus expression vector (see Annual Report 2000-01). This vector was developed by the University of Florida Vector Core. We have optimized labeling embryonic Schwann cells and routinely achieve transduction efficiencies greater than 90%. After transplantation into host nerves, GFP-expressing cells were labeled using anti-GFP immunohistochemistry, providing an effect means to track the growth and migration of the (prelabeled) cells after transplantation into the host nerve. The aims of Task 1 were completed.

Task 2: Engraft Nf1 Schwann cells in Nf1^{+/+} and Nf1^{-/+} host nerves:

Progress:

Thus far we have engrafted 64 nerves with GFP-labeled *Nf1* or wild-type Schwann cell cultures. Various conditions were tested and grafts were examined after periods ranging from 6-84 days. *Nf1*^{-/-} and *Nf1*^{-/+} Schwann cells were engrafted in wild-type mice and mice with a *Nf1*^{-/+} background. GFP-labeled cells were identified in nearly every engrafted nerve. However, after short time periods (6-28 days) cell numbers were general quite low, well below the number injected originally (10⁵). Quantitative estimates based on serial sections indicated that less than 1,000 cells were present in any given nerve and more than one-half of the nerve grafts contained less than 100 GFP-labeled cells. This was true for both *Nf1*^{-/-} and *Nf1*^{-/+} Schwann cells implants. There was some indication that cell numbers were higher when implants were made into mice with an *Nf1* background. Nevertheless, the overwhelming conclusion was that the syngrafted cells did not grow well and no large tumor-like formations were observed in any of the nerves, even after 12 weeks (Annual Report 2001-02).

In search of an explanation for the failure of the syngraft to grow, we examined the proliferation of cells in the mouse nerves. After engraftment, mice were given systemic injections of the DNA analog bromodeoxyuridine (BrdU). Several days later, the nerves were removed and then immunostained for BrdU-DNA (marker for proliferation) and GFP (marker for implanted cells). In all cases we observed proliferating (BrdU-positive) cells amongst the GFP labeled cells, indicating that at least some of the implanted cells were multiplying. This suggests that the cells were viable and capable of proliferating in the nerve. Nevertheless, it was apparent that the number of transplanted cells did not increase and, instead, cell loss was prevalent. Two explanations seemed most likely; either the cells were dying or their expression of the GFP was falling off. We first believed that GFP expression was stable and persistent in vivo (see Annual Report 2001-02). This was based on findings that transfected Schwann cells continue to express GFP for many months in culture and that AAV-GFP transduction of neurons in vivo can persist for well over six months. Also, in many of the nerves where only a few engrafted cells are seen, these show a strong GFP signal. Our recent evidence, however, indicates GFP expression by the implanted cells does indeed wane over time in vivo (see below). The other possibility is that the AAV-GFP transfected Schwann cells are evoking an immune response and are being actively rejected by the host. This could be due to subtle differences in the Nf1 and wild-type B6 mouse strains or perhaps the expression of AAV proteins as a result of prelabeling the cells. To circumvent this problem we

engrafted the NfI^{-/-} and NfI^{-/+} Schwann cells into immunodeficient scid mice, with and without an NfI background. Initial results (see Annual Report 2001-02) from short-term implants into the scid mice did not show any major improvements in growth by the implanted Schwann cells. However, we have now completed the analysis of nerve implants grown for up to six months. These results show extensive tumor growth in nerves implanted with Nf1-- Schwann cells. The tumor masses showed a high number of proliferating cells (BrdU-positive) and these were stained by the Schwann cell marker, S-100. Also, GFP-positive cells were scattered throughout these cell masses, but the vast majority did not label for GFP. In contrast, nerves implanted Nf1^{-/+} Schwann cells contained no conspicuous cell masses, very few proliferating cells and GFP-labeled cells were scarce. Furthermore, almost no GFP-labeled cells were found in nerves implanted with wild-type Schwann cells and overall these nerves appeared normal. These results indicate that Nf1^{-/-} Schwann cells proliferated slowly but persistently forming conspicuous masses in the nerves of scid mice after 6 months, whereas Nf1^{-/+} and wild-type Schwann cells did not. It appears that the GFP expression by the implanted cells indeed diminished over time and thus only a small percentage of the implanted cells could be tracked by this method after prolonged growth in vivo. From these findings we conclude that NfI^{-/-} Schwann cells show tumorigenic growth and that syngraft of Nf1--- Schwann cells into scid mice may be a valuable mouse model for neurofibroma. The aims of Task 2 were completed. The analysis of additional nerve implants in the long-term studies have not yet been completed. These will provide the number of test animals required to judge the statistical significance of these findings.

Task 3: Test neonatal engraftment and nerve injury paradigms:

Progress:

We investigated various neonatal injection methods and found the use of very young mice to be impractical. See Annual Report 2001-02 for details. Another strategy to hasten the growth of implanted cells was to induce a proliferative response by nerve injury and we concluded that nerve injury did not improve the growth of the implanted cells (see Annual Report 2001-02). The aims of task were completed. In summary, we now know that proliferation by $NfI^{-/-}$ cells is growth factor independent and thus the intended purpose for nerve injury (inducing the release of Schwann cell growth factors) might not be effective for these cells. In addition, nerve injury may not be feasible to hasten tumor growth because the formation of tumors by $NfI^{-/-}$ Schwann cells requires many months and nerve repair in mice is complete within a much shorter time frame.

Task 4: Examine nerve grafts for tumorigenic growth and statistically analyze data:

Progress:

As described above, we have examined the growth of wild-type, $NfI^{-/+}$, and $NfI^{-/-}$ Schwann cells after implantation into the mouse nerve. Cell numbers were assessed by counting GFP-labeled cells in serial sections of the engrafted nerves. For the short-term implants, the data were categorized into 4 groups (no cells, 1-10, 11-100, more than 100 cells). No grafts contained more than 1,000 cells, indicating that in all grafts less than 1% of the injected cells were present 1-12 after injection. We concluded that there was a rapid initial dying off of the injected cells. Data indicated that on average 0.1% of the cells survived for up to 12 weeks. As stated above, we recently found that $NfI^{-/-}$ Schwann cells form sizeable tumor-like masses after 6 months when implanted in the nerves of *scid* mice. To date

we have analyzed only 4 mice (8 nerves) in this condition (all of which showed sizeable tumors). Additional mice have been implanted and will be examined in the near future. At that point the data analyses will be performed.

Task 5: Examine growth of engrafted Schwann cells by in vivo NMR.

Progress:

NMR imaging was not performed on the syngrafts because of the lack of tumor formation in our initial studies. Our efforts focused exclusively on the NMR of xenografts (see below).

Technical Objective 2: DETERMINE THE TUMORIGENIC PROPERTIES OF HUMAN PLEXIFORM NEUROFIBROMA-DERIVED SCHWANN CELLS ENGRAFTED IN MOUSE NERVE.

Task 1: Breed mouse strain crossing *Nf1* knockout mice and immunodeficient *scid* mice:

Progress:

Protocols for rapid and reliable genotyping were established for NfI knockout and scid (immunodeficient) genotypes. Breeding colonies of these strains are stable and maintenance breeding is routine. Cross-breeding was successful and a stable population of $NfI^{-/+}/scid$ breeding pairs is maintained and litters achieved as needed. Adequate numbers of $NfI^{-/+}/scid$ animals are available for grafting experiments. The aims of Task 1 were competed.

Task 2: Engraft human plexiform tumor Schwann cells in $NfI^{+/+}$ and $NfI^{-/+}$ host nerves:

Progress:

The tumorigenic growth of selected human neurofibroma Schwann cell (NF1-SC) cultures was first examined as xenografts in the sciatic nerves of adult immunodeficient *scid* mice (with no *Nf1* background). Each NF1-SC culture was engrafted into 4 nerves and 6 nerves were engrafted with an equal number of normal human SC. Engrafted nerves were examined after 8 weeks by immunostaining with an antibody specific to human glutathione s-transferase (GST) (see Annual Report 2000-01). These results demonstrate sustained tumor growth by neurofibroma-derived human SC implanted in the mouse nerve (Muir et al., 2001). Additional proliferation properties were reported in a related publication (Fieber et al., 2003). Our general conclusions are that tumor development for all cell lines was very slow and that more than 8 weeks would be required to achieve sizeable tumor masses.

Because of the need for such long tumor growth periods, we also examined the in vivo growth of Schwann-like cell lines from two malignant peripheral nerve sheath tumors (MPNST) established previously in our lab. It is notable that both MPNST lines share many properties with the more transformed plexiform Schwann cell lines. We initially found that only one of the MPNST lines consistently developed sizeable tumors within 8 weeks of engraftment into the nerves of *scid* mice. We have now achieved rapid and reliable growth by several MPNST lines in the xenograft model. These tumors were very reminiscent of plexiform neurofibromas and did not metastasize. Importantly, detailed examination of these tumors with vascular and angiogeneic markers revealed significant neovascularization. To date we have examined more than 40 nerves with extensive tumors in this model.

We also tested the hypothesis that the NfI background plays an important role in the tumorigenic growth by these NF1 cell lines. We have now engrafted 40 nerves (in both scid and $NfI^{-/+}/scid$) with these cell line for periods ranging from 2 to 24 weeks. MPNST Schwann cells were engrafted into mice with a Nf1 background ($NfI^{-/+}/scid$) and tumors growth for each cell line was examined after 2 weeks (n=8), 8 weeks (n=8) and 24 weeks (n=8) and compared to tumor growth in mice without the Nf1 background ($NfI^{+/+}/scid$). Quantitative analysis indicated that the Nf1 background plays a marginal role in the growth rate and size of these tumors. However, interesting and more substantial differences in the cell composition and vascular elements were observed. These considerations are now the basis for aims in our new project funded by the U.S. Army. The aims of Task 2 were completed.

Task 3: Test neonatal engraftment and nerve injury paradigms:

Progress:

As stated in Technical Objective 1, Task 3, we investigated various neonatal injection methods and found the use of very young mice to be impractical and that nerve injury was not effective in our original design. In regard to the present aim, we have achieved rapid and sustained growth by the implantation of the more highly tumorigenic NF1MPNST cell lines. Accordingly, these strategies to hasten tumor growth are not necessary in our improved xenograft model. The aims of Task 3 were complete.

Task 4: Examine growth of human tumor grafts by *in vivo* NMR:

Progress:

To examine the growth of tumors grafts in vivo by NMR it was necessary first to examine the imaging and contrast parameters of normal and pathological nerve. These experiments led to significant progress to enhance the imaging of nerve components (see Annual Report 2000-01). We have now performed NMR on numerous engrafted nerves, with excellent results. The contrast imaging of tumor growing within the nerve has been performed using T1, T2, and diffusion-weighted methods. Serial slices through the nerves provided a full representation of the extent of tumor growth. Thereafter, the nerves were examined histologically and we confirmed the NMR profiles were indeed representative of the distribution and density of the engrafted tumor cells. For volumetric quantitation were are creating 3D renderings of the NMR data. In parallel, we are creating 3D renderings of the histological images as well. This alleviates the any concerns about differences in imaging alignment (the imaging plane) between the NMR sections and the histology sections. It also provides the ability to reconstruct the entire volume of tumor growth for quantitative correlations between the two imaging methods. Furthermore, volumetric data will be the method of choice for assessing the extent of tumor growth and regression in this model when applied to testing of anti-tumor therapies. Most of the initial NMR data was obtained using ex vivo nerves and we have now obtained excellent results imaging tumors in the living animal, including the use of contrast enhancement agents for examining the vascular elements and blood flow within the tumor grafts. Our results in these efforts have been very successful and we conclude that NMR imaging can be successfully applied to the quantitation of tumor growth in this mouse tumor model. The aims of Task 4 were completed.

Task 5: End-point histology assessment of tumor grafts and statistically analyze data:

Progress:

All tumor grafts, regardless of the amount of tumor growth, have been examined by routine histology and immunohistochemical staining. Human NF1 tumor cells have been clearly identified using the human-specific antibody to GST (a constitutively expressed marker protein). This labeling has provided a reliable assessment of neurofibroma growth and cell invasion. Tumors have also been labeled with a human-specific antibody to Ki-67, a proliferating cell marker, to estimate the proliferation of engrafted cells. As expected from overall tumor size, most of the plexiform tumors contain few Ki-67-positive cells, confirming that the GST-positive cells are surviving in the mouse nerve but proliferate very slowly. On the other hand, the MPNST-derived Schwann cell grafts show a much higher level of Ki-67 labeling and thus mitotic index. These labeling techniques will be exploited in all subsequent experiments, particularly those to examine methods to increase tumor growth (e.g., in neonatal implantation and nerve injury models).

An important measure of tumor development is its ability to attract blood vessels, or neovascularize. We have made excellent progress in examining the tumors for microvessel formation and key molecular components in this response. All well-developed neurofibroma grafts contained CD-31/von Willebrand factor (factor VIII-related antigen)-positive capillaries. These tumors also labeled intensely for both VEGF and the VEGF receptor, suggesting active recruitment of endothelial cells and vessel formation. These findings will be important for the assessment of tumor growth and in correlations with naturally occurring neurofibromas. In addition, our long-term goal is to use this mouse model to test anti-angiogenic therapies for neurofibroma. The aims of Task 5 were completed.

KEY RESEARCH ACCOMPLISHMENTS

- 1) Established and characterized numerous Schwann cell cultures from Nf1 knockout mouse embryos.
- 2) Found that Nf1^{-/-} Schwann cells are growth factor-independent and have a growth advantage in vitro.
- 3) Effectively transfected Nf1^{-/-} Schwann cell cultures with AAV-GFP.
- 4) Found Nf1^{-/-} cells have tumorigenic properties and form tumors by 6 months in scid mouse nerves.
- 5) Established an reliable model for Nf1 tumors by syngraft of Nf1^{-/-} Schwann cells.
- 6) Established a breeding colony of $NfI^{-/+}/scid$ mice (a unique strain).
- 7) Characterized the tumorigenic properties of numerous human NF1 tumor Schwann cell cultures.
- 8) Found sizeable, invasive tumors result from engrafting human NF1 Schwann cells in mouse nerves.
- 9) Established a reliable and rapid xenograft model for NF1 tumors.
- 10) Achieved effective NMR imaging of tumors in the xenograft tumor model.
- 11) Determined tumorigenic growth and neovascularization by engrafted NF1 tumor cells.

REPORTABLE OUTCOMES

New Funding based on the progress of this project

DAMD17-03-1-0224 (Muir)
Dept of Defense Army Medical and Materiel Command
Angiogenesis and therapeutic approaches to NF1 tumors

05/01/03 - 04/30/07 \$1,548,903 total award

Manuscripts

- D. Muir, D. Neubauer, I.T. Lim, A.T. Yachnis, M.R. Wallace. 2001. The tumorigenic properties of neurofibromin-deficient Schwann cell lines subcultured from human neurofibromas. *American Journal of Pathology* 158: 501-513.
- L.A. Fieber, D.M. González, M.R. Wallace, D. Muir. 2003. K channel blockers inhibit proliferation in NF1 Schwann cells. *Neurobiology of Disease* 13: 136-146.
- Y. Li, P.K. Rao, R. Wen, D. Muir, M.R. Wallace, S. van Horne, G.I. Tennekoon, T. Kadesch. 2003. Activated Notch1 transforms Schwann cells. *Oncogene* (in press)

Abstracts of Presentations

- G. Perrin, M. Wallace, A. Edison and D. Muir. 2001. Neurofibromin-deficient Schwann cell xenografts as a model of plexiform neurofibroma. Society for Neuroscience Annual Meeting, San Diego, CA
- G. Perrin, M. Wallace and D. Muir. 2001. Neurofibromin expression may not directly correlate to NF1 tumorigenesis. National Neurofibromatosis Foundation Meeting, Aspen, CO.
- M. Wu, G. Perrin, M. Wallace, D. Muir. 2002. Development of mouse models of NF1 tumorigenesis. National Neurofibromatosis Foundation Meeting, Aspen, CO.
- G. Perrin, R. Walton, T. Mareci and D. Muir. 2002. Using NMR to monitor tumor formation and progression in a mouse model of neurofibromatosis type I plexiform neurofibroma. Society for Neuroscience Annual Meeting, Orlando, FL
- L. Feiber, M. Wallace, D. Muir. 2002. K channel blockers inhibit proliferation in NF1 Schwann cells. Society for Neuroscience Annual Meeting, Orlando, FL
- G. Perrin, M. Wallace and D. Muir. 2003. Characterization of a reproducible xenograft model for NF1 plexiform neurofibroma. National Neurofibromatosis Foundation Meeting, Aspen, CO.
- M. Wu, M. Wallace, D. Muir. 2003. Tumorigenic growth properties of syngrafted Nf1 knockout Schwann cell. National Neurofibromatosis Foundation Meeting, Aspen, CO..
- S.A.M. Thomson, L. Fishbein, D. Muir, M.R. Wallace. 2003. Genetic analysis of NF1 plexiform tumor cultures by cDNA expression arrays. National Neurofibromatosis Foundation Meeting, Aspen, CO.

Animal Resources

New mouse immunodeficient mouse strain with the Nf1 background, Nf1-/+/scid.

CONCLUSIONS

Excellent progress was made in establishing $NfI^{-/-}$ Schwann cell cultures. We documented heightened proliferative properties in these cells suggesting that Nf1-deficiency increases their tumorigenic potential. $NfI^{-/-}$ Schwann cell cultures were effectively prelabeled with AAV-GFP which has proven to be a useful marker for cells implanted in the mouse nerve for up to several months, but not for longer periods. Significant tumorigenic growth by $NfI^{-/-}$ implants required up to six months in immunodeficient scid mice hosts. We tested several adjunctive methods to hasten tumor growth, but none were successful. Based on these data, we conclude that embryonic $NfI^{-/-}$ Schwann cell have a moderate in vivo tumorigenic potential and that these cells form tumors very slowly, like that of human NF1 neurofibromas. Although this data is informative and indicative of the tumorigenic properties of Nf1 deficient cells, the syngraft model is difficult and time-consuming for routine use in testing antitumor therapies for neurofibromas.

A thorough characterization of neurofibromin-deficient plexiform tumor-derived cell lines was published. This article included the first report of a xenograft model showing tumorigenic growth by human neurofibroma Schwann cells transplanted in the mouse nerve. Although most of the neurofibroma xenografts develop very slowly we also developed a more rapidly growing model of neurofibroma using a Schwann cell line derived from a MPNST. Large tumors were grown in the mouse nerve and these were examined for tumorigenic growth, neovascularization and several key molecular components in this process. NMR has proven to be an effective means to assess tumor growth and may be used for volumetric quantitation to examine the effects of anti-cancer therapies. Taken together, we have developed and characterized a valid and very practical xenograft model for neurofibroma including a non-invasive and clinically relevant method for monitoring tumor progression. This animal model is easily adopted by other laboratories and immediately applicable to the testing of anti-cancer therapies for neurofibroma.

REFERENCES

- D. Muir, D. Neubauer, I.T. Lim, A.T. Yachnis, M.R. Wallace. 2001. The tumorigenic properties of neurofibromin-deficient Schwann cell lines subcultured from human neurofibromas. *American Journal of Pathology* 158: 501-513.
- L.A. Fieber, D.M. González, M.R. Wallace, D. Muir. 2003. K channel blockers inhibit proliferation in NF1 Schwann cells. *Neurobiology of Disease* 13: 136-146.

PAID PERSONNEL

David Muir, Margaret Wallace, Thomas Mareci, Eileen Monk, Min Wu, Anthony Yachnis, James Graham, Robert Walton, Craig Krekoski

APPENDICES

1 article:

Fieber et al., 2003. Neurobiology of Disease 13: 136-146.

5 abstracts:

Perrin et al., 2001a

Perrin et al., 2001b

Perrin et al., 2002

Wu et al., 2002

Perrin et al., 2003

Note: The following article was appended to Annual Report 2000-01.

Muir et al., 2001. American Journal of Pathology 158: 501-513.



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Delayed rectifier K currents in NF1 Schwann cells Pharmacological block inhibits proliferation

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Abstract

K+ (K) currents are related to the proliferation of many cell types and have a relationship to second messenger pathways implicated in regulation of the cell cycle in development and certain disease states. We examined the role of K currents in Schwann cells (SC) cultured from tumors that arise in the human disease neurofibromatosis type 1 (NF1). Comparisons were made between whole cell voltage clamp recordings from normal human SC cultures and from neurofibroma cultures and malignant peripheral nerve sheath tumor (MPNST) cell lines. The outward K currents of normal and tumor cells could be divided into three types based on pharmacology and macroscopic inactivation: (1) "A type" current blocked by 4-aminopyridine, (2) delayed rectifier (DR) current blocked by tetraethylammonium, and (3) biphasic current consisting of a combination of these two current types. The DR K current was present in MPNST- and neurofibromaderived SC, but not in quiescent, nondividing, normal SC. DR currents were largest in MPNST-derived SC (50 pA/pF vs. 2.1-4.9 pA/pF in dividing and quiescent normal SC). Normal SC cultures had significantly more cells with A type current than cultures of MPNST and the plexiform neurofibroma. Conversely, MPNST and plexiform neurofibroma cultures had significantly more SC with DR current than did normal cultures, and these DR currents were significantly larger. In addition, the plexiform neurofibroma culture had significantly more cells with DR current than the dermal neurofibroma culture. K currents in SC from normal NF1 SC cultures had current abundances similar to GGF-exposed normal SC and the plexiform neurofibroma. We have established a link between DR K current blockade via TEA analogs and inhibition of proliferation of NF1 SC in vitro. In addition, a farnysyl transferase inhibitor (FTI), a blocker of Ras activation, blocked cell proliferation without blocking K currents in all cultures except a plexiform neurofibroma, suggesting that regulation of proliferation in neoplastic and normal SC in vitro is complex. © 2003 Elsevier Science (USA). All rights reserved.

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Keywords: Tumor; Patch clamp; Human; Neurofibroma; K current; Delayed rectifier

Introduction

Although the human neurofibromatosis type 1 (NF1) gene has been cloned (Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990) and its protein product, neurofibromin, identified (Xu et al., 1990), the function of neurofibromin and its role in tumorigenesis remains enigmatic. Neurofibromin is a normal constituent of the cell cytoplasm, with significant expression after embryogenesis limited to

cells of neuroectodermal origin (Daston et al., 1992a, 1992b), such as neurons and Schwann cells (SC). While the NF1 gene appears to function as a tumor suppressor gene, contributing to inactivation of the cellular proto-oncogene ras, the role of neurofibromin in cell proliferation appears complex and has been difficult to dissect in SC. For example, the critical amount of neurofibromin for normal cell function is unknown. In addition, increased Ras activity leads to SC differentiation, not proliferation (Kim et al., 1995). These observations have led to consideration of additional intracellular elements contributing to the NF1 cellular phenotype besides neurofibromin and Ras. Experiments to explore the function of neurofibromin and its

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modulation in animal models have resulted in several physiological studies implicating the intracellular signaling molecules cyclic AMP (cAMP) and protein kinase C in defining the phenotype of NF1-deficient cells (Guo et al., 1997; Kim et al., 1997a, 1997b; Ratner et al., 1998). In two studies, second messengers were found to control K channel function. In Drosophila (Guo et al., 1997), where neurofibromin appears to be strongly associated with the function of adenylyl cyclase, a muscle K current dependent on intact adenylyl cyclase and Ras signaling was nonfunctional in NF1-deficient files, implying that the absence of neurofibromin interfered with normal adenylyl cyclase activity. The link between neurofibromin and adenylyl cyclase was corroborated in mouse neurofibromin-deficient SC, which have increased K current density compared to SC from wild-type controls (Xu et al., 2002). Inhibitors of protein kinase A significantly decreased the K channel density of mouse neurofibromin-deficient SC. Inhibition of Ras with a dominant-negative Ras had a potentiating effect on K current density.

While the ultimate goal of NF1 research is to find appropriate therapies, considerable research remains to be done to understand the cellular physiology of this disease. An important component of SC physiology, which has remained relatively unknown in NF1, is the electrophysiology of affected cells and the relationship of ion currents to development and maintenance of the NF1 SC phenotype. Recent studies have demonstrated that K currents are different in normal human SC and in SC derived from tumors in the diseases NF1 (Fieber, 1998) and NF2 (Kamleiter et al., 1998; Rosenbaum et al., 2000).

Voltage-gated K currents constitute the main conductances found in SC, although SC also have Na⁺ currents and, in mouse, Ca²⁺ currents (Amedee et al., 1991). The K currents of SC are (1) inward-rectifier (IR) K current that conducts K⁺ out of the cell in response to hyperpolarizing voltage stimuli, (2) time inactivating, A-type current, an outward K current that is blocked preferentially by 4-aminopyridine (4-AP), and a complex, composite current called delayed rectifier (DR) current, which is an outward conductance that is blocked by tetraethylammonium (TEA). DR currents are composed of many different molecular entities that can be present alone or in various combinations to produce the observed current properties. DR channels of SC are represented by the four families Shaker, Shaw, Shal, and Shab and include homo- and heteromultimers of many channel subunits including Kv1.1, Kv1.2, Kv1.5, Kv2.1, Kv3.1, and Kv3.2 (Sobko et al., 1998).

In the present study we examined the relationship between a K channel current, which may be unique to SC derived from tumors, and SC proliferation. Evidence suggests that these K currents are directly related to the proliferative capacity of many cell types and have a relationship to well-characterized second messenger pathways that play a role in the cell cycle during development and in certain disease states (Rane, 1999). By studying the K currents of

NF1 SC and their relationship to proliferation, we hope to provide insights about the tumorigenic process in NF1.

Materials and methods

Cell culture

Normal cauda equina from human organ donors were obtained with full legal consent from the family of the donor by the University of Miami Organ Procurement Team. The cauda equina were harvested within 1 h of aortic clamping and stored in Belzer's cold storage solution at 4° C for ≤ 3 days prior to placement in culture (Levi et al., 1995).

Fresh (never cryopreserved) primary cultures of normal SC obtained from 4 donors constituted the "PW" series of cell cultures, generously provided by Dr. Patrick Wood. Nerve fascicles were freed of connective tissue and superficial blood vessels, cut into 2-mm-long explants, and the explants placed in 35-mm dishes containing D culture medium consisting of Dulbecco's modified Eagle's medium (DMEM; Life Sciences Technologies, Grand Island, NY), 10% heat inactivated fetal bovine serum with 50 U/ml penicillin, and 0.05 mg/ml streptomycin. In 3 of the 4 PW normal SC cultures, the D medium was supplemented with 1 μM forskolin and 10 nM recombinant human heregulin (rh-heregulin, also termed glial growth factor (GGF)); Casella et al., 1996) to stimulate SC proliferation. No growth factors were added to the fourth PW culture (PW1). After 2 weeks, dissociated SC were obtained by 18 h of enzymatic digestion of the explant in 0.25% dispase (Boehringer Mannheim Biochemicals, Indianapolis, IN), 0.05% collagenase (Worthington Biochemical Corporation, Freehold NJ), and 15% fetal bovine serum (Hyclone, Logan, UT) in D medium. The SC were plated on collagen-coated 35-mm dishes and maintained at 37°C in a 6% CO2 atmosphere. Cultures prepared in this way have been shown to contain 92% pure SC by S-100 staining (Casella et al., 1996). The normal culture not exposed to GGF and forskolin was purified by successive platings for short periods on tissue culture plastic dishes before plating on collagen coated dishes. This culture was approximately 90% SC, and was studied 10 days after dissociation. One to 10 days before use in experiments, normal cultures were transferred to normal culture medium at 37°C in a 5% CO₂ atmosphere. Normal culture medium consisted of 85% DMEM (with high glucose and pyruvate), 15% fetal bovine serum, and 50 U/ml penicillin, 0.05 mg/ml streptomycin, and 2 mM glutamine (all from Life Sciences Technologies), without forskolin or

The "DM" neurofibroma and malignant peripheral nerve sheath tumor (MPNST) cultures, and DM normal SC cultures were obtained from human donors and cultured according to Muir et al. (2001) to enrich for SC, similarly to the procedure described above for PW SC cultures. The MPNST SC line T265 was obtained as frozen stock from

Dr. George De Vries (Badache and De Vries, 1998). All MPNST lines were derived from patients with NF1. DM9 and DM10 SC cultures were derived from the normal nerves of different NF1 patients, either from limb amoutation or post mortem. The nerves from which these cultures were derived were not associated with tumors, nor did histology show neoplastic elements. We refer to these cultures as "normal SC cultured from NF1 patients." Prior to electrophysiological recordings and proliferation studies, DM and T265 cultures were plated from cryopreserved stocks. All of the subcultures and tumor cells lines described in this section were maintained in normal culture medium described above, on poly-L-lysine- and laminin-coated 75-mm² plastic tissue-culture dishes. In this medium, tumor cells divided rapidly and became confluent within days of subculture. Subcultures were plated onto poly-L-lysine- and laminincoated 35-mm Falcon dishes. Poly-L-lysine and laminin were obtained from Sigma.

Electrophysiological methods and analysis

Whole cell currents were recorded using the whole cell variation of the patch clamp technique with patch pipettes of 0.5-2 M Ω . Electrophysiological experiments were performed on isolated cells in sparsely seeded cultures that were not confluent. Recordings were limited to bipolar cells in cultures known to contain other cell types besides SC. The intracellular solution contained (mM): 150 KCl, 1 ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 CaCl₂, 5 MgCl₂, 5 Na₂ATP, and 40 N-[2hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES)-KOH, pH 7.0. The calculated intracellular free Ca²⁺ concentration was ~40 pM (Owen, 1976). Normal extracellular solution (ECS) consisted of (mM): 170 NaCl, 3 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 10 HEPES-NaOH, pH 7.25. 4-Aminopyridine solution (4-AP) was made by adding 5 mM 4-AP (Sigma) to ECS and readjusting the pH to 7.25; 50 mM tetraethylammonium (TEA) solution was made by adding TEACl (Sigma) to ECS with a decreased NaCl content. The cells were continuously perfused with extracellular solutions via 1-µL pipettes attached to gravity-dispensed solution reservoirs.

Current amplitudes were directly comparable even though the size of cells varied, because the currents (in picoamperes, pA) were normalized to the cell's capacitance (in picofarads, pF).

Currents were recorded at room temperature (19–22°C) with an Axopatch 200A or 200B using the PClamp 6 programs (Axon Instruments, Foster City, CA) and a 400 MHz PC with a Digidata 1200 A-D converter (Axon Instruments). Electrophysiological data were 4-pole low-pass Bessel filtered and digitized at 10 kHz. The capacitive current cancellation method of Howe and Ritchie (1990) with 80% series resistance compensation was used, with voltage drops resulting from uncompensated capacitance (<3 mV) not subtracted from the data records. In prelimi-

nary experiments no differences in SC currents present were observed when the chloride channel blocker 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB; 100 μ M) was included in the ECS. Thus, it was assumed that chloride channels did not contribute substantially to the outward currents observed.

Neurofibromin assays

Neurofibromin Western immunoblotting was performed as in Muir et al. (2001), where the neurofibromin status of the DM cultures used in this study was first reported. The dermal and plexiform neurofibroma cultures and the MPNST cultures were neurofibromin-negative, except for 1 MPNST (DM6) that had a variant (and possibly nonfunctional) form. Full-length neurofibromin was undetectable in two SC cultures derived from the normal nerves of different NF1 patients. Normal cultures were either determined to be neurofibromin positive, or were not tested but assumed neurofibromin positive. The neurofibromin-negative status of MPNST culture T275 was reported by Badache et al. (1998).

Proliferation assays

SC were plated at 12,000 per 35-mm dish and five fields per 35-mm dish in three replicate dishes were counted at 200× magnification. Doubling times were determined by dividing the average of cell counts at $t_{0+(54 \text{ to } 72)h}$ by average cell counts obtained at t_0 , where $t_0 = 24$ h after plating, then normalizing to 2. The bromodeoxyuridine (BrdU) immunoassay (Zymed) was used to assess proliferation of SC in culture. SC cultures were exposed to BrdU for 5-6 h. Immunolabeling for proliferating cell nuclear antigen (PCNA) was used as a proxy for cell division in selected cells in which K currents were previously recorded. PCNA indicates cells that were in the G2 phase of the cell cycle at the time they were fixed. K currents were recorded with lucifer yellow (1 mg/ml; Sigma-Aldrich) in the patch pipette, then the cultures were fixed and the PCNA assay (Zymed) was run. Cells were identified by means of lucifer yellow fluorescence, then the PCNA status of these identified cells was assessed under phase-contrast optics.

The effects of several reagents on proliferation and K currents were studied. TEA analogs tetrapentylammonium (TPeA; 50 μ M) and tetrahexylammonium (THeA; 5 μ M; Sigma-Aldrich) were used to block K currents. A farnesyl transferase inhibitor (FTI; FPT Inhibitor III, Calbiochem; 10 and 50 μ M; Kim et al., 1997b) was used to block Ras. Cells plated on 18- or 25-mm round glass coverslips for proliferation assays and onto the bottoms of 35-mm dishes for electrophysiology were exposed for 48–72 h to these individual reagents. To determine the number of stained cells and total cell counts, five fields of cells per coverslip were counted in three replicate dishes exposed to one of

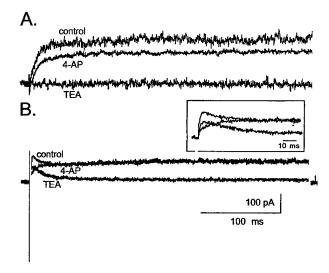


Fig. 1. K currents in tumor Schwann cells (SC). (A) Pharmacological experiments at 20 mV from a holding potential of -70 mV in an MPNST cell to identify the K currents as delayed rectifier (DR) currents, preferentially blocked by tetraethylammonium (TEA). This cell has a small current component blocked also by 4-aminopyridine (4-AP). Traces show control currents and currents after bath application of TEA, then after washout and subsequent bath application of 4-AP. MPNST, malignant peripheral nerve sheath tumor. (B) Pharmacological experiments at 20 mV in a plexiform neurofibroma SC to identify the biphasic K currents. This cell has a DR component blocked only by TEA, and an A type component blocked only by 4-AP. The insert shows the initial phase of these currents on an expanded scale with the TEA-exposed trace as a heavy line.

these agents, in addition to unexposed control cultures and cultures to which no primary antibody or BrdU was added.

Statistical analysis

Data were analyzed for frequency of occurrence using the G test (Sokal and Rohlf, 1995), and for average differences using Student's t test (Datadesk for the Macintosh, Ithaca, NY).

Results

Overview

Results from assays for cell proliferation and electrophysiology presented in the Tables and Figures were obtained on early passages of normal and NF1 cell cultures, except for MPNST T265 where the passage number was high. Cultures were maintained for extended periods only to assess the durability of proliferation rates with time away from GGF or forskolin. All cell cultures were assumed to remain SC enriched for the brief time they were kept in culture to produce electrophysiological and proliferation results. This assumption was supported by morphological observations on the cell cultures. In addition, data were

acquired from cells with clearly distinguishable SC morphology.

While we have classified these tumors in individual categories according to the histological type of neurofibroma, because these are human tumors and not samples from inbred animals, considerable variation was expected in tumor composition and histology. In some tumor cultures, all SC had the same currents. In other cultures there was some degree of cell-to-cell variation. Some cells had more than one K current type. When two K current types co-occurred in a single cell, they produced a biphasic current that could be dissected using component-specific blockers (see Fig. 1B). Thus, we characterized the distribution of the currents both as components of a biphasic current and as "pure" currents.

Normal and neurofibroma-derived SC currents

Studies on NF1 cell cultures and cell lines confirm the findings of Fieber (1998) in NF1 MPNST cell lines. The most conspicuous difference between normal and tumorderived SC K currents in culture was that tumor cells functionally expressed DR currents (Fig. 1A), while quiescent, normal SC had either no outward K currents or only A type currents (Fig. 2). Na⁺ currents and inward rectifying K⁺ currents were occasionally observed in recordings from both normal and tumor-derived cultured SC, as in Fieber (1998).

SC currents and proliferation status

Quiescent normal SC never exposed to GGF (PW1) studied soon after dissociation of the nerve (Levi et al.,

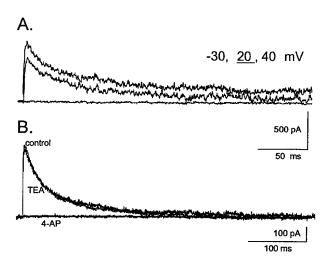


Fig. 2. K currents in normal Schwann cells (SC). (A) Family of K currents to the indicated test potentials from a holding potential of -70 mV. (B) Pharmacological experiments at 20 mV to identify the K currents as A type preferentially blocked by 4-aminopyridine (4-AP). Traces show control currents and currents after bath application of tetraethylammonium (TEA), then after washout and subsequent bath application of 4-AP.

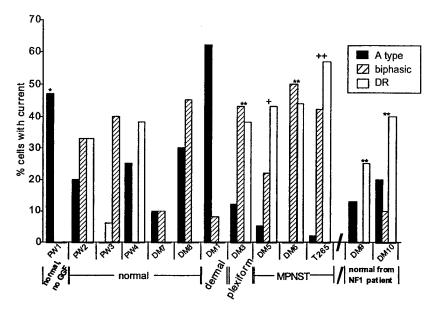


Fig. 3. K current abundances by type. Normal culture PW1 was purified without glial growth factor (GGF) or forskolin, while all other cultures were exposed to GGF during their purification and in some cases prior to electrophysiological experiments. *A type current abundance significantly different from A currents of MPNSTs and DM8, G test, $P \le 0.05$. **, ****, *, *+*DR plus biphasic current abundances significantly different from these current abundances in: **Normal culture PW1 and dermal neurofibroma (DM1), $P \le 0.05$. Current abundances in DM9 and DM10 cultures combined for this analysis. *Normal culture PW1 and dermal (DM1) and plexiform (DM3) neurofibromas, $P \le 0.05$. **Normal cultures PW1 and DM7, $P \le 0.05$. MPNST, malignant peripheral nerve sheath tumor; DR, delayed rectifier.

1995) had small amplitude outward K currents of the transient, A type, blocked by 4-AP (Fig. 2A and B; Fieber, 1998). Forty-seven percent of quiescent normal SC had A

type currents averaging 4.84 ± 0.54 pA/pF (Figs. 3 and 4). Other quiescent normal SC had no outward currents, although some had IR K currents or Na⁺ currents. Many

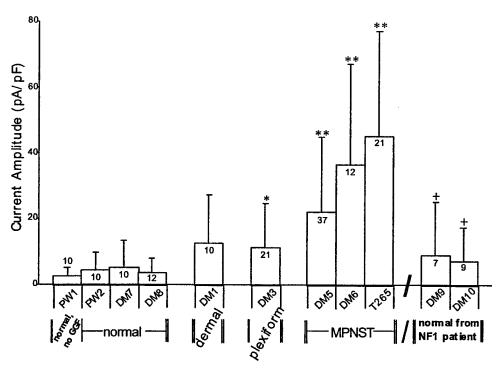


Fig. 4. Maximum current amplitudes without selection for current type, +SD. Numbers above or inside bars are sample size. *Significantly different from MPNSTs and the plexiform neurofibroma culture DM8, t test, $P \le 0.05$. **Significantly different from all normal cultures, and from dermal and plexiform neurofibromas, $P \le 0.05$. *Significantly different from MPNSTs, $P \le 0.05$. MPNST, malignant peripheral nerve sheath tumor.

Table 1 Summary of outward K currents in normal and NF1 SC^a

SC culture type	Predominant outward K current(s)	Significant trends
Normal Dermal neurofibroma Plexiform neurofibroma MPNST	A type A type, biphasic Biphasic, noninactivating Noninactivating	Noninactivating increases A type increases

^a SC, Schwann cells; MPNST, malignant peripheral nerve sheath tumor.

normal SC do not have recorded currents in vitro (Fieber, 1998).

Normal SC cultures maintained in GGF + forskolin exhibited doubling times of 38-87 h and had A type and biphasic currents, plus DR currents of small amplitude (2.1-3.0 pA/pF; Figs. 3 and 4). They occasionally had Na⁺ currents.

Normal SC 6 days after withdrawal of GGF proliferated in culture in 15% serum on laminin-coated dishes, with doubling times of 38–87 h. K currents of dividing normal SC were A type or DR currents (1.4–12.2 pA/pF), or biphasic K currents composed of A type and DR currents, respectively (Figs. 3 and 4). Other currents, such as Na⁺ currents, were much rarer.

The SC of all tumor cultures plated on laminin divided in culture medium containing 15% serum but no GGF. Doubling times in the neurofibroma culture and the MPNST cell lines after 1 week to 7 months in culture were ~50 h.

The hallmark K current of tumor-derived SC cultures was the DR current that was blocked by TEA (Fig. 1A). The DR K current was present in ~50% of all MPNST SC and in 38% of plexiform neurofibroma SC (Fig. 3). A type, biphasic, and Na⁺ currents were also observed, as were, rarely, IR currents. The mean amplitudes of DR K currents of different tumor SC cultures ranged at 10–50 pA/pF, with the largest DR currents occurring in MPNSTs (Fig. 4). Details of the K currents of NF1 tumor cultures are presented below.

K currents in all the *MPNST* cell lines were classical, DR currents (Fig. 1A). Different current profiles were seen in the dermal and plexiform neurofibromas. Some SC from the plexiform neurofibroma had DR or biphasic currents (Figs. 1B and 3). All K currents in cells derived from a dermal neurofibroma resembled those of proliferating normal SC, with A type or biphasic K currents, but no pure tumor DR currents.

Differences in current abundance shown for the A type and DR type (including biphasic) currents are shown in Fig. 3. Normal SC culture PW1, never exposed to growth factors, had significantly more cells with A type current than cultures of MPNST and the plexiform neurofibroma, and, conversely, MPNST and plexiform neurofibroma cultures had significantly more SC with DR currents than did normal culture PW1. In addition, the plexiform neurofibroma culture had significantly more cells with DR current than the dermal neurofibroma culture. K currents in SC from normal

NF1 SC cultures, though variable within a culture, had current abundances similar to GGF-exposed normal SC and the plexiform neurofibroma.

Fig. 4 shows the average maximum current amplitude in each culture, without selection for current type. Current amplitude was an additional important difference between the DR currents of normal SC and those of tumor SC. In normal, dividing SC the K currents present, whether DR, A type, or biphasic, were smaller in amplitude than in any tumor cell culture. Mean K current amplitudes of normal SC cultures were significantly different than mean current amplitudes of all MPNST cell cultures and the plexiform neurofibroma culture. K current amplitudes in the dermal neurofibroma were not significantly different from those in any normal SC culture.

Normal SC cultures from NF1 patients

Two SC cultures derived from the normal nerves of different NF1 patients had a doubling time of 63 h after 14 days withdrawn from GGF, and their K current abundances were like normal proliferating SC (Fig. 3). As a pooled sample, these normal NF1 SC cultures from NF1 patients had significantly more DR currents than both the normal culture never exposed to GGF and the dermal neurofibroma, but were not different from any MPNST culture, the plexiform neurofibroma, or proliferating normal cultures withdrawn from GGF. In addition, the amplitude of the DR current of the pooled sample of normal SC cultures from NF1 patients was significantly different from that of MPNST cultures but not the proliferating normal cultures (Fig. 4).

The K current types formed a continuum from normal SC to malignant tumor SC, with A type K current found in normal SC and SC from the dermal neurofibroma, and noninactivating K current found in the more malignant tumor cultures (Table 1).

TEA analogs block SC proliferation and SC K currents

The significant differences in abundance and amplitude of DR currents between normal and tumor-derived SC suggested experiments to investigate a possible relationship between DR currents and proliferation in NF1, as found in NF2 (Rosenbaum et al., 2000), and other proliferating cells (Knutson et al., 1997; Liu et al., 1998). The classical blocker

Table 2
Effect of TEA analogs on cell proliferation and electrophysiological parameters in normal, MPNST- and neurofibroma-derived SC^a

Tissue source	Name	TEA analogs ^b		
		% inhibition of proliferation ^c	Change in K amplitude	Decrease in RP (mV)
Normal nerve	DM7	100 ± 0%	ND	ND
	PW4	ND	22% decreaseg	ND
MPNST	DM5	$100 \pm 0.03\%$	73% decreased	24 ^e
	DM6	$96.5 \pm 2.6\%$	97% decrease ^{e,f}	13 ^d
	T265	94 ± 6%	96% decreased	26e
Dermal neurofibroma	DM1	$71.5 \pm 28\%$	ND	ND
Plexiform neurofibroma	DM3	$100 \pm 0\%$	>82% decrease ^d	12e
Normal nerve of NF1 patient	DM10	$100 \pm 0\%$	ND	ND

^a TEA, tetraethylammonium; MPNST, malignant peripheral nerve sheath tumor; SC, Schwann cells; ND, not determined; THeA, tetrahexylammonium; TPeA, tetrapenthylammonium.

of DR currents is TEA. We exposed SC cultures to two analogs of TEA that do not appear to be toxic to cells: TPeA (50 μ M) and THeA (5 μ M; Wilson and Chiu, 1993). After 54 h, cell proliferation was assayed. In 3 MPNST cell lines, 2 neurofibroma SC cultures, and 1 normal, dividing SC culture, these agents completely or almost completely inhibited tumor SC proliferation (Table 2). We verified that THeA and TPeA, also blocked K currents in tumor cells at the same concentrations that blocked proliferation, either severely reducing or abolishing recorded whole cell K currents compared to matched controls: (same cell passage; Table 2). The current block washed out within minutes after 24-h exposure to TEA analogs, confirming that THeA and TPeA blocked currents by the conventional manner of a channel plug rather than by causing downregulation of channel expression. Cells exposed to TEA analogs for up to 48 h appeared morphologically similar to controls, with a membrane sufficiently intact for electrophysiological experiments. After 72 h in 5 µM THeA, membrane seals were more difficult to obtain.

The control resting potential (RP) of SC in different MPNST- and neurofibroma-derived SC cultures averaged -26 to -44 mV. TEA analogs applied for >40 h depolarized RPs by >50% of their control values (Table 2). The reduced RPs observed in TPeA and THeA were significantly different from controls. Within 1 h of washout of TEA analogs (and relief of DR channels from block), RPs were not significantly different from matched controls (data not shown).

Proliferation and K currents were monitored in the cell line T265 after 54-h incubation in normal culture medium with 10 mM added KCl to test the idea that the inhibition of proliferation was a secondary consequence of the change in RP caused by TEA analogs. This procedure for reducing RP should have little effect on voltage-gated K channels. Al-

though 10 mM added KCl reduced the RP of T265 cells by an average of 23 mV, the same amount of depolarization caused by TEA analogs in this cell line, it was without effect on proliferation rates or K current amplitude (n = 8; data not shown).

Proliferation in a normal SC culture also was blocked by TEA analogs; however, average K channel amplitude was not significantly different after 40-h exposure to these agents (Table 2). A type current is not as susceptible to block by chronic exposure to TEA analogs as DR currents. Because the DR current component was small relative to the A type component in these cells (Fig. 2B), the blockade of DR current of normal SC did not significantly alter the average K channel current.

Effects of blocking ras on proliferation and currents

Neurofibromin inactivates Ras, thereby acting as a tumor suppressor. The effects of blocking Ras on functional expression of different K channel-types were studied via application of a membrane-permeant farnesyl transferase inhibitor (FTI) to tumor and proliferating normal SC.

FTI was added to the normal culture medium of cells for 2 days before BrdU proliferation assays and/or electrophysiological experiments in tumor and normal SC cultures and one normal NF1 SC culture. Controls were passage- and time-matched cultures. In all MPNST, neurofibroma, and normal cultures tested, FTI inhibited proliferation (Table 3). FTI did not revert the tumor cells to a normal morphological phenotype (Yan et al., 1995). 48 hr FTI-exposed cultures had cell counts comparable to t_0 cell counts.

Although significant depolarizations of the RP occurred in some FTI-exposed MPNST-derived SC cultures, the size of the K currents was not significantly different for any tumor SC exposed to FTI except the plexiform neurofi-

^b 5 μM THeA and 50 μM TPeA treatments were approximately equal in their effects; therefore, experiments using either were pooled.

^c Mean ± standard error.

^d Significantly different from control (t test, P < 0.05).

^e Significantly different from control (t test, P < 0.01).

f THeA only.

g Not a significant decrease.

Table 3
Effect of farnesyl transferase inhibitor (FTI) on cell proliferation in normal, MPNST-, and neurofibroma-derived SC^a

Tissue source	Name	FTI		
		$10 \mu M (\% \text{ inhibition of proliferation})^b$	50 μ M (% inhibition of proliferation) ^b	
Normal nerve	DM7	$87.5 \pm 0.9\%$	100 ± 0%	
	PW2	$86 \pm 0\%$	ND	
MPNST:	DM5	$58 \pm 0\%$	94 ± 6%	
	DM6	64 ± 19%	$99 \pm 0.5\%$	
	T265	$2 \pm 0\%$	69 ± 0%	
Plexiform neurofibroma	DM3	81 ± 0%	$100 \pm 0\%^{c}$	
Normal nerve of NF1 patient	DM10	$95 \pm 0\%$	ND	

^a MPNST, malignant peripheral nerve sheath tumor; SC, Schwann cells; ND, not determined.

broma. FTI did not cause obvious changes in the percent composition of DR, A type, and biphasic currents.

Characterizing K currents of dividing vs. resting cells

Despite significant differences in current abundance and amplitude between cultures, both normal and tumor SC cultures showed interculture variation in K currents, which may have some relationship to the cell cycle. To identify changes in K currents that correlate with changes in the cell cycle, we made electrophysiological recordings from cells, fixed them, then examined whether these specific cells expressed PCNA at the time the recordings were made. Experiments were performed on the plexiform neurofibroma SC and a normal SC culture that was proliferating. The PCNA status of 9 plexiform neurofibroma cells and 10 normal SC was evaluated after locating the cells via staining with lucifer yellow that had been injected through the recording pipette. When the cells from both cultures were grouped together most PCNA-negative (nonproliferating) cells had A type or biphasic K currents. Most PCNApositive SC had DR currents (Table 4). The frequency of pure DR currents had a significant correlation with a positive PCNA status (P = 0.05; G test).

Discussion

These results extend a previous report (Fieber, 1998) describing DR K currents in SC of MPNST cells lines to

Table 4
K currents in pooled neurofibroma and normal SC as a function of proliferation status^a

Proliferation status	% cells with A type or biphasic current	% cells with DR current	
Proliferating	11	21	
Nonproliferating	57	11	

a SC, Schwann cells.

low-passage-number subcultures of neurofibromas and MPNSTs. DR currents were characteristic of tumor cultures while A type currents were more frequently observed in quiescent normal SC. The variation of K current types in dermal and plexiform neurofibroma cultures, and the similarities of some of their K currents to those present in normal SC raises the possibility that SC ion channels from at least some types of neurofibromas are not physiologically abnormal or that the ion channel phenotypes of normal, neurofibroma-derived, and MPNST-derived SC represent a continuum. Molecular characterization of the channel types present would elucidate this; however, because SC DR currents arise from channels consisting of complex heteromultimers of at least eight known subunits whose arrangement is unknown (Sobko et al., 1998), this is a challenging task with uncertain likelihood of success. The channel differences are as likely to occur in the abundance of subunit types present as in their presence or absence, whereas only the latter can be assessed using the two available techniques of subtype-specific antibodies or analysis of transcripts corresponding to specific subunits.

Normal SC cultures subcultured in the presence of mitogens such as GGF and forskolin failed to eventually revert to a quiescent state and maintained high proliferation rates weeks after removal from these substances. Sustained cell proliferation has been observed previously in SC stimulated with growth factors (Langford et al., 1988), and attributed to a permanent effect of growth factors on the cell cycle of SC, or an induced predisposition of SC to divide in response to factors present in serum or that SC produce. In our study, serum or laminin might have acted to promote sustained cell proliferation, because these cultures were plated on laminincoated dishes, and laminin has been found to be mitogenic for SC (McGarvey et al., 1984; Muir et al., 1989).

The SC of the neurofibroma-derived cultures and all MPNST-derived cultures used in this study continue to divide and maintain their S-100-positive status for many weeks after withdrawal of GGF and forskolin (Muir et al., 2001). This is characteristic of many cells derived from

^b Mean ± standard error.

 $^{^{\}rm c}$ Inhibition by 50 μ M FTI resulted in 85% decrease in K current amplitude.

tumors, and it focuses interest on normal cultures derived from NF1 patients. If these SC are growth factor independent, they provide an important intermediate category of SC. The normal cultures derived from NF1 patients proliferated when plated on laminin in contrast to many other neurofibroma-derived SC cultures that remained quiescent under these culture conditions (Muir et al., 2001).

K channels play prominent roles in controlling proliferation and differentiation in a variety of inexcitable cell types (Knutson et al., 1997; Liu et al., 1998), and have been implicated in the growth of tumor cells (Rane, 1999; Stringer et al., 2001). Studies of cell cycle control mechanisms have demonstrated that the K currents characteristic of proliferating cells are involved in induction of proliferation, rather than being a byproduct of this process (Nilius et al., 1993; Jones et al., 1995). This suggests that K channels generally are involved in one of the signaling process associated with cell division.

The close relationship between K channels and SC proliferation has been well described in other systems. Studies of K currents of SC in animal models have demonstrated that large outward DR K currents are characteristic of proliferating SC of the embryonic nervous system (Konishi, 1990; Chiu and Wilson, 1989; Pappas and Ritchie, 1998). Pharmacological block of the DR K current of proliferating SC results in suppression of proliferation (Chiu and Wilson, 1989; Wilson and Chiu, 1993; Pappas and Ritchie, 1998).

We now extend these findings of proliferation associated with development to postembryonic human SC. TEA analogs blocked human SC DR K channels and also blocked proliferation of SC, whether the SC were cultured from tumors or normal nerves. In normal SC, the blockade of DR current did not significantly alter the average K channel current. This provides additional evidence that the DR rather than the A type current is the component associated with proliferation in human SC. These data suggest that DR channels have a role in proliferation of both normal and NF1 SC in vitro and that the SC K channel profile observed may depend in part on the proliferation status of the cells. This latter idea is supported by the results of our lucifer yellow experiments, in which DR currents were positively correlated with cells in G2 phase. These data corroborate those of Rosenbaum et al. (2000) on NF2 schwannomaderived SC in vitro in which the IC₅₀ for inhibition of proliferation by quinidine, which blocks K channels in NF2 SC, was 25 μ M. Thus, it appears that K channels have a role in the control of proliferation in postembryonic human SC.

Neurofibromin-positive, proliferating normal SC had, at most, small DR currents while DR currents were common in four cultures derived from plexiform neurofibroma or MPNST. These results suggest that there is a fundamental difference in K currents of NF1 tumor SC that may not be related specifically to proliferation, but instead to their derivation from NF1 tumors. Such a role would be expected to coincide with an absence of the tumor suppressor protein neurofibromin. Accordingly, the plexiform neurofibroma-

derived culture and two MPNST-derived cultures lacked full-length neurofibromin and the other MPNST assessed for neurofibromin expression had a variant (and possibly nonfunctional) form. However, no pure DR K currents were found in a fourth neurofibromin-negative culture (the dermal neurofibroma). Thus, it is important to consider the alternative hypothesis that it is not total DR K current size that is associated with the absence of neurofibromin, but rather a particular component of DR current. Pharmacological tools that identify other components of DR K currents could be used to address this hypothesis.

In addition to TEA analogs, an FTI also blocked proliferation in normal and tumor cells, but it did not affect DR currents of most cultures (the exception was a plexiform neurofibroma culture). In NF1, neurofibromin levels in tumor-derived SC are abnormally reduced or absent, theoretically freeing Ras from persistent inactivation by this tumor suppressor (Rutkowski et al., 2000; Muir et al., 2001). Accordingly, Ras-GTP levels are high in MPNST cell lines (DeClue et al., 1992). Drugs such as FTI inhibit Ras proteins in tumor SC by preventing a specific posttranslational modification that promotes their association with the plasma membrane, which is essential to their activation (Lowy and Willumsen, 1993). Thus FTI, while not restoring normal neurofibromin levels, inactivates Ras. In a plexiform neurofibroma, our results suggest that inhibition of proliferation is related to inhibition of both Ras and of DR channels. But this is clearly not the case in any MPNST cell line or in normal SC. In these latter cells, FTI-induced inhibition of proliferation occurs without affecting K channels. One interpretation for this observation is that some Ras proteins act on the cell cycle independently from K channels. Since not all cellular Ras is activated by farnesylation, FTI may knock out sufficient Ras to inhibit proliferation, but not all Ras within SC, specifically sparing those Ras proteins that are linked to K channels. In addition, FTI may affect other proteins besides Ras (Gibbs, 2000), some of which, like ERK-1 and 2, may play a role in the cell cycle (Lewis et al., 1998) but may not be linked to K channels. Finally, K channel modulation by Ras-activated pathways is probably sufficiently complex that blocking Ras alone may not revert the electrophysiological characteristics of tumor cells to normal. Furthermore, there may be significant differences in the control of the cell cycle by Ras proteins and K channels in different NF1 tumor types (DeClue et al., 1992). Additional studies of proliferation and K channel block in NF1 tumor SC are required with specific inhibitors of other Ras proteins as well as inhibitors of other intracellular messengers implicated in the Ras-initiated cascade such as Raf, Ras-mitogen activated protein kinase (MAPK) and MAPK kinase (MEK).

Although TEA analogs significantly depolarized SC RP, depolarization is probably not the cause of inhibition of proliferation by these agents. The RP of a cell is determined primarily by the difference in K⁺ concentration on either side of the membrane, as maintained by the different routes

for K⁺ entry, such as ion channels, the Na⁺/K⁺ ATPase, and leak conductances. Kodal et al. (2000) proposed an explanation for how K channel block can cause depolarization. When IR K channels are present at very low density, the opening or closure of only a few DR K channels can cause large (tens of mV) fluctuations of the RP. Few active IR channels cause the cells to become depolarized, which drives open DR K channels. DR channel opening hyperpolarizes the RP again, but if the DR channels are blocked, the RP remains depolarized.

Few studies have addressed the physiological aspects of the altered relationship between neurons and glia that occurs in NF1 tumorigenesis. Among the symptoms of NF1 are assumed electrophysiological changes in the function of the central nervous system that lead to learning and motor deficits (Eldridge et al., 1989; Pensak et al., 1989). These CNS alterations are inarguably complex. Yet a new study has demonstrated that broad manipulations to lower Ras levels in a mouse model of NF1 reverse learning deficits by blocking inhibitory potentials mediated by GABA-activated ion channels (Costa et al., 2002). Therefore, the relationships between ion channels of the nervous system, and Ras, neurofibromin, or other second messengers such as MEK and MAPK are an appropriate focus of studies to understand the different changes occurring in NF1, including tumorigenesis.

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References

- Amédée, T., Elie, B., Dupouy, B., Vincent, J.D., 1991. Voltage-dependent calcium and potassium channels in Schwann cells cultured from dorsal root ganglia of the mouse. J. Physiol. 441, 35-56.
- Badache, A., De Vries, G.H., 1998. Neurofibrosarcoma-derived Schwann cells overexpress platelet derived growth factor (PDGF) receptors and are induced to proliferate by PDGF BB. J. Cell Physiol. 177, 334-342.
- Badache, A., Muja, N., De Vries, G.H., 1998. Expression of Kit in neurofibromin-deficient human Schwann cells: role in Schwann cell hyperplasia associated with type 1 neurofibromatosis. Oncogene 17, 795–800.
- Casella, G.T.B., Bunge, R.P., Wood, P.M., 1996. Improved method for harvesting human Schwann cells from mature peripheral nerve and expansion in vitro. Glia 17, 327-338.
- Cawthon, R.M., Weiss, R., Xu, G., Viskochil, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland, R., O'Connell, P., White, R., 1990. A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. Cell 62, 193-201.

- Chiu, S.Y., Wilson, G.F., 1989. The role of potassium channels in Schwann cell proliferation in Wallerian degeneration of explant rabbit sciatic nerves. J. Physiol. (Lond.) 408, 199–222.
- Costa, R.M., Federov, N.B., Kogan, J.H., Murphy, G.G., Stern, J., Ohno, M., Kucherlapati, R., Jacks, T., Silva, A.J., 2002. Mechansism for the learning deficits in a mouse model of neurofibromatosis type 1. Nature 415, 526-530.
- Daston, M.M., Ratner, N., 1992a. Neurofibromin, a predominantly neuronal GTPase activating protein in the adult, is ubiquitously expressed during development. Dev. Dynam. 195, 216–226.
- Daston, M.M., Scrable, H., Nordlund, M., Sturbaum, A.K., Nissen, L.M., Ratner, N., 1992b. The protein product of the neurofibromatosis type 1 gene is expressed at highest abundance in neurons, Schwann cells, and oligodendrocytes. Neuron 8, 415–428.
- DeClue, J.E., Papageorge, A.G., Fletcher, J., Diehl, S.R., Ratner, N., Vass, W.C., Lowy, D.R., 1992. Abnormal regulation of mammalian p21ras contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. Cell 69, 265–273.
- Eldridge, R., Denckla, M.B., Bien, E., Myers, S., Kaiser-Kupfer, M.I., Pikus, A., Schlesinger, S.L., Parry, D.M., Dambrosia, J.M., Zasloff, M.A., Mulvihill, J.J., 1989. Neurofibromatosis type 1 (Recklinhausen's disease). Neurologic and cognitive assessment with sibling controls. Am. J. Dis. Child. 143, 883–837.
- Fieber, L.A., 1998. Ionic currents in normal and neurofibromatosis type 1-affected human Schwann cells: induction of tumor cell K current in normal Schwann cells by cyclic AMP. J. Neurosci. Res. 54, 495-506.
- Gibbs, J.B., 2000. Anticancer drug targets: growth factors and growth factor signaling. J. Clin. Invest. 105, 9-13.
- Guo, H.-F., The, I., Hannan, F., Bernards, A., Zhong, Y., 1997. Requirement of Drosophila NF1 for activation of adenylyl cyclase by PACAP38-like neuropeptides. Science 276, 795–798.
- Howe, J.R., Ritchie, J.M., 1990. Sodium currents in Schwann cells from myelinated and non-myelinated nerves of neonatal and adult rabbits. J. Physiol. (Lond.) 425, 169-210.
- Jones, S.M., Hofmann, A.D., Lieber, J.L., Ribera, A.B., 1995. Overexpression of potassium channel RNA: in vivo development rescues neurons from suppression of morphological differentiation in vitro. J. Neurosci. 15, 2867–2874.
- Kamleiter, M., Hanemann, C.O., Kluwe, L., Rosenbaum, C., Wosch, S., Mautner, V.F., Muller, H.-W., Grafe, P., 1998. Voltage-dependent membrane currents of cultured human neurofibromatosis type 2 Schwann cells. Glia 24, 313–322.
- Kim, H., Rosenbaum, T., Marchionni, M., Ratner, N., DeClue, J., 1995. Schwann cells from neurofibromin deficient mice exhibit activation of p21^{ras}, inhibition of cell proliferation and morphological changes. Oncogene 11, 325–335.
- Kim, H.A., DeClue, J.E., Ratner, N., 1997a. cAMP-dependent protein kinase A is required for Schwann cell growth: interactions between the cAMP and neuregulin/tyrosine kinase pathways. J. Neurosci. Res. 49, 236–247.
- Kim, H.A., Ling, B., Ratner, N., 1997b. Nf1-deficient mouse Schwann cells are angiogenic and invasive and can be induced to hyperproliferate: reversion of some phenotypes by an inhibitor of farnesyl protein transferase. Mol. Cell. Biol. 17, 862–872.
- Knutson, P., Ghiani, C.A., Zhou, J.M., Gallo, V., McBain, C.J., 1997. K⁺ channel expression and cell proliferation are regulated by intracellular sodium and membrane depolarization in oligodendrocyte progenitor cells. J. Neurosci. 17, 2669–2682.
- Kodal, H., Weick, M., Moll, V., Biedermann, B., Reichenbach, A., Bringmann, A., 2000. Involvement of calcium activated potassium channels in the regulation of DNA synthesis in cultured Müller glial cells. Invest. Ophthalmol. Vis. Sci. 41, 4262–4267.
- Konishi, T., 1990. Voltage-gated potassium currents in myelinating Schwann cells in the mouse. J. Physiol (Lond.) 431, 123–139.
- Langford, L.A., Porter, S., Bunge, R.P., 1988. Immortalized rat Schwann cells produce tumours in vivo. J. Neurocytol. 17, 521–529.

- Levi, A., Bunge, R., Lofgren, J., Meima, L., Hefti, F., Nikolics, K., Sliwkowski, M., 1995. The influence of heregulins on human Schwann cell proliferation. J. Neurochem. 15, 1329-1340.
- Lewis, T.S., Shapiro, P.S., Ahn, N.G., 1998. Signal transduction through MAP kinase cascades. Adv. Cancer Res. 74, 49-139.
- Liu, S.I., Chi, C.W., Lui, W.Y., Mok, K.T., Wu, C.W., Wu, S.N., 1998. Correlation of hepatocyte growth factor-induced proliferation and calcium-activated potassium current in human gastric cancer cells. Biochim. Biophys. Acta 1368, 256–266.
- Lowy, D.R., Willumsen, B.M., 1993. Function and regulation of ras. Annu. Rev. Biochem. 62, 851–891.
- McGarvey, M.L., Baron-Van Evercooren, A., Kleinman, H.K., Dubois-Dalcq, M., 1984. Synthesis and effects of basement membrane components in cultured rat Schwann cells. Dev. Biol. 105, 18-28.
- Muir, D., Gennrich, C., Varon, S., Manthorpe, M., 1989. Rat sciatic nerve Schwann cell microcultures: responses to mitogens and production of trophic and neurite-promoting factors. Neurochem. Res. 14, 1003– 1012.
- Muir, D., Neubauer, D., Lim, I.T., Yachnis, A.T., Wallace, M.R., 2001.
 Tumorigenic properties of neurofibromin-deficient neurofibroma
 Schwann cells. Am. J. Pathol. 158, 501–513.
- Nilius, B., Schwarz, G., Droogmans, G., 1993. Control of intracellular calcium by membrane potential in human melanoma cells. Am. J. Physiol. 265, C1501-C1510.
- Owen, J.D., 1976. The determination of the stability constant for calcium-EGTA. Biochem. Biophys. Acta 451, 321–325.
- Pappas, C.A., Ritchie, J.M., 1998. Effect of specific ion channel blockers on cultured Schwann cell proliferation. Glia 22, 113-120.
- Pensak, M.L., Keith, R.W., Dignan, P.S., Stowens, D.W., Towbin, R.B., Katbamna, B., 1989. Neuroaudiologic abnormalities in patients with type 1 neurofibromatosis. Laryngology 99, 702-705.
- Rane, S.G., 1999. Potassium channel as targets for modulating cell growth and differentiation. Perspect. Drug Discov. Design 15/16, 295-311.
- Ratner, N., Bloom, G.S., Brady, S.T., 1998. A role for cyclin-dependent kinase(s) in the modulation of fast anterogrde axonal transport: effects defined by olomoucine and the APC tumor suppressor protein. J. Neurosci. 18, 7717-7726.
- Rosenbaum, C., Kamleiter, M., Grafe, P., Kluwe, L., Mautner, V.-F., Müller, H.W., Hanemann, C.O., 2000. Enhanced proliferation and

- potassium conductance of Schwann cells isolated from NF2 Schwannomas can be reduced by quinidine. Neurobiol. Dis. 7, 483-491.
- Rutkowski, J.L., Wu, K., Gutmann, D.H., Boyer, P.J., Legius, E., 2000. Genetic and cellular defects contributing to benign tumor formation in neurofibromatosis type 1. Hum. Mol. Genet. 9, 1059-1066.
- Sobko, A., Peretz, A., Shirihai, O., Etkin, S., Cherpanova, V., Dagan, D., Attali, B., 1998. Heteromultimeric delayed-rectifierr K+ channels in Schwann cells: developmental expression and role in cell proliferation. J. Neurosci. 19, 10398-10408.
- Sokal, R.R., Rohlf, F.J., 1995. Biometry. W.H. Freeman, San Francisco.Stevens, B., Tanner, S., Fields, R.D., 1998. Control of myelination by specific patterns of neural impulses. J. Neurosci. 18, 9303–9311.
- Stringer, B.K., Cooper, A.G., Shepard, S.B., 2001. Overexpression of the G-protein inwardly rectifying potassium channel 1 (GIRK1) in primary breast carcinomas correlates with Axillary lymph node metastasis. Cancer Res. 61, 582-588.
- Viskochil, D., Buchberg, A.M., Xu, G., Cawthon, R.M., Stevens, J., Wolff, R.K., Culver, M., Carey, J.C., Copeland, N.G., Jenkins, N.A., White, R., O'Connell, P., 1990. Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. Cell 62, 187–192.
- Wallace, M.R., Marchuk, D.A., Andersen, L.B., Letcher, R., Odeh, H.M., Saulino, A.M., Fountain, J.W., Brereton, A., Nicholson, J., Mitchell, A.L., Brownstein, B.H., Collins, F.S., 1990. Type 1 neurofibromatosis gene: Identification of a large transcript disrupted in three NF1 patients. Science 249, 181–186.
- Wilson, G.F., Chiu, S.Y., 1993. Mitogenic factors regulate ion channels in Schwann cells cultured from newborn rat scatic nerve. J. Physiol. (Lond.) 470, 501-520.
- Xu, G.F., O'Connell, P., Viskochil, D., Cawthon, R., Robertson, M., Culver, M., Dunn, D., Stevens, J., Gesteland, R., White, R., Weiss, R., 1990. The neurofibromatosis type-1 gene encodes a protein related to GAP. Cell 62, 599-608.
- Xu, Y., Chiamvimonvat, N., Vázquez, A.E., Akunuru, S., Ratner, N., Yamoah, E.N., 2002. Gene-targeted deletion of neurofibromin enhances the expression of a transient outward K⁺ current in Schwann cells: a protein kinase A-mediated mechanism. J. Neurosci. 22, 9194– 9202.
- Yan, N., Ricca, C., Fletcher, J., Glove, T., Seizinger, B., Manne, V., 1995. FTP inhibitors block the neurofibromatosis type 1 (NF1) malignant phenotype. Cancer Res. 55, 3569-3575.

G. Perrin, M. Wallace and D. Muir. 2001. Neurofibromin expression may not directly correlate to NF1 tumorigenesis. National Neurofibromatosis Foundation Meeting, Aspen, CO.

Neurofibromas and malignant peripheral nerve sheath tumors (MPNST) arise frequently in neurofibromatosis type 1 (NF1). The two-hit hypothesis of tumor suppressor genes predicts deficiency of functional neurofibromin as a major determinant in neurofibroma development. Neurofibromas are composed of heterogeneous cell populations including Schwann cells, fibroblastic cells and possible intermediates or variants. We examined the cellular expression of neurofibromin in NF1 tumors, non-tumoral (normal) nerves, and derivative Schwann cell cultures. Immunocytochemical staining shows that most neurofibromas contain populations of neurofibromin-negative and neurofibrominpositive Schwann cells (S100-positive cells). We subcultured Schwann cells from many such tumors and found these cells to be neurofibromin-deficient, as indicated by western immunoblotting. We examined neurofibromas and normal nerves (obtained at autopsy or amputation) from two severely afflicted NF1 patients. In these cases, plexiform and dermal tumors were consistently devoid of neurofibromin-labeled Schwann or fibroblastic cells. Remarkably, the normal NF1 nerves (taken from distant nerves deemed histologically normal) were sporadically labeled compared to control (non-NF1) nerves and contained mostly unlabeled Schwann cells, although some neurofibromin-positive Schwann cells and other nerve elements were clearly evident. These findings were confirmed by western immunoblotting of derivative Schwann cell cultures in which full-length neurofibromin was undetectable. Thus neurofibromin deficiency may not be limited to overt tumors. Interestingly, we find that a number of NF1 MPNSTs contain predominantly neurofibromin-positive cells and full-length neurofibromin is abundant in primary cultures of these malignant tumors. Continuous cell lines have been established from MPNSTs with variable Schwann cell features. Most of these, as well as several other MPNST lines obtained from other labs, express abundant full-length neurofibromin (as determined by western immunoblotting using several anti-peptide antibodies). These findings raise important questions about reconciling the two-hit premise and genetic data with protein-based analyses and characterizing the extent of neurofibromin deficiency in NF1 tumorigenesis.

G. Perrin, M. Wallace, A. Edison and D. Muir. 2001. Neurofibromin-deficient Schwann cell xenografts as a model of plexiform neurofibroma. Society for Neuroscience Annual Meeting, San Diego, CA

Plexiform neurofibromas are peripheral nerve sheath tumors that arise frequently in neurofibromatosis type 1 and have a risk of malignant progression. Past efforts to establish animal models for human neurofibromas have relied on the implantation of tumor fragments and heterogeneous primary cultures. The goal of the present study was to develop an animal model by intraneural xenograft of neurofibromin-deficient human Schwann cells (SC). Highly enriched SC cultures were established from 8 plexiform neurofibromas and 2 neurofibrosarcomas by selective subculture using glial growth factor-2 and laminin. These cultures were shown to be neurofibromin-deficient by Western blot analysis. Engraftment of neurofibromin-deficient SC cultures into the peripheral nerves of scid mice consistently produced persistent neurofibroma-like tumors with diffuse and often extensive intraneural growth. Like the human tumors, growth by engrafted plexiform-derived SCs was quite slow and variable, and thus was inconvenient for certain study aims. On the other hand, the neurofibrosarcoma grafts showed moderate proliferation and more rapidly infiltrated and remodeled the host nerve. Furthermore, the histogenesis of engrafted neurofibrosarcomas was very consistent with that of established human plexiform neurofibromas. These intraneural engraftment models are the first to achieve tumorigenic growth in vivo by human neurofibromin-deficient SC and provides the means to study the histogenesis of neurofibromas in a relevant cellular environment. A detailed comparison of these neurofibroma models with human neurofibroma is underway.

G. Perrin, R. Walton, T. Mareci and D. Muir. 2002. Using NMR to monitor tumor formation and progression in a mouse model of neurofibromatosis type I plexiform neurofibroma. Society for Neuroscience Annual Meeting, Orlando, FL

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Plexiform neurofibromas in patients with neurofibromatosis type I (NF1) typically involve deep or named nerves, can become very large, may cause serious functional impairment and have a risk of malignant progression. In developing an animal model to study plexiform neurofibromas, we have shown that human NF1 neurofibrosarcomaderived Schwann cells (SC) can be successfully xenografted into the sciatic nerves of scid mice. The histogenesis of engrafted neurofibrosarcoma SC was consistent with that of established human plexiform neurofibromas. The tumors invaded and degenerated the host nerve structure and proliferated in a slow, sustained manner without apparent malignancy. In addition, we observed angiogenic markers in xenografted nerves. We performed MRI to study the formation and progression of these tumors in our model. Using a 17.6 tesla, 89mm bore magnet we performed H-1 MRI at 750MHz on excised, fixed xenografted mouse sciatic nerves. We then compared the T1 weighted images to immunostaining of the same nerves with an antibody specific to human glutathione Stransferase. The hypointense areas of nerve seen in the MRI data corresponded to the areas staining positive for human tumor cells. Similar to human plexiform neurofibromas, demyelinization and increased extracellular matrix were also observed and likely contributed to the effects seen in the MRI data. Therefore, we can use MRI to monitor tumor development and progression. We are currently using MRI to further investigate tumor progression and angiogenesis in vivo.

M. Wu, G. Perrin, M. Wallace, D. Muir. 2002. Development of mouse models of NF1 tumorigenesis. National Neurofibromatosis Foundation Meeting, Aspen, CO

Past efforts to establish animal models for neurofibroma have relied on generating transgenic mice or the implantation of tumor fragments or heterogeneous primary cultures. The goal of the present study was to develop animal models by intraneural xenograft of neurofibromin-deficient human Schwann cells (SC) and by syngraft of mouse Nf1-/- SC both into scid/Nf1-/+ mice. Xenograft of human NF1 SC showed moderate proliferation and extensive infiltration of the host nerve. This model is the first to achieve intraneural tumorigenic growth by human neurofibromin-deficient SC and provides the means to study the histogenesis of neurofibromas in a relevant cellular environment. To develop a syngraft mouse model, we have successfully enriched SC cultures from Nf1knockout mice. From our preliminary results, we observed higher cell proliferation rate from NF1-/- culture than wild type. These SCs were labeled with a marker protein using AAV vector mediated GFP transduction. Expression of GFP was detected from SCs in vitro cell culture and in vivo engrafted nerve. Comparing with Nfl-/+ SCs, Nf1 knockout SCs shown better persistence in host nerve although any tumorlike growth was quite slow. Detailed studies of SC proliferation in vitro and in viro, as well as engrafted tumor development and comparisons with human neurofibroma is underway.

G. Perrin, M. Wallace and D. Muir. 2003. Characterization of a reproducible xenograft model for NF1 plexiform neurofibroma. National Neurofibromatosis Foundation Meeting, Aspen, CO.

Several genetic mouse models of neurofibromatosis type 1 (NF1) have been developed recently to study tumorigenesis by neurofibromin-deficient Schwann cells. We have developed a mouse xenograft model in which human NF1 tumor-derived cell cultures are implanted into the nerves of immunodeficient mice with an Nf1+/- background. This xenograft model has several advantages. First, the xenograft model takes advantage of the numerous primary (mixed cell type) and Schwann cell enriched cultures established from dermal, plexiform and malignant (including primary, recurrent, and metastases) tumor cultures, as well as Schwann cell cultures we have established from normal nerves from NF1 and non-NF1 patients (see Muir et al, Am J Pathol, 2001, 158:501-513). Second, prior to implantation, cultures can be examined for in vitro neoplastic properties, karyotype and genetic abnormalities. Third, the investigator can precisely define the initiation of tumor xenoplants by cell number, time and location in a relevant cellular environment. Fourth, xenoplants can be initiated in hosts at various developmental stages and with various genetic and phenotypic alterations. On the other hand, xenografting requires the use of immunodeficient mice and can complicate the interpretation of hostimplant cell interactions. However, it is clear that this model recapitulates the main aspects of tumor growth including tumor cell proliferation, invasion, nerve disruption and demyelination as well as infiltration of host mast cells and endothelial cells in angiogenesis. Similar to neurofibromas in patients, growth by most xenoplants is slow and many months are required for substantial tumor development. Here we describe the implantation of neurofibrosarcoma cell lines that form plexiform-like tumors rapidly and reproducibly. Extensive characterizations of the cell lines and histological and MRI analyses of tumorigenic growth as xenografts are presented. The use of permanent cell lines, a newly established scid/Nf1+/- mouse strain, and standard methodology provide for high reproducibility by different laboratories. Our goal is to establish and communicate a valid working model for comparable study and testing of therapeutic approaches for NF1 tumors.